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Determination of Mycotoxins in Cereal-Based Porridge Destined for Infant Consumption by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract An analytical method applying ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) for the determination of aflatoxins M2, M1, G2, G1, B2, B1, deoxynivalenol, ochratoxin A, fumonisins B1 and B2, hydrolyzed fumonisins B1 and B2, zearalenone and sterigmatocystin in cereal-based porridge destined for infant consumption was developed and validated. The mycotoxins were extracted using an adequate solvent ratio (n-hexane/3% formic acid solution/acetonitrile) under rapid shaking and sonication, without any further cleanup steps. Recoveries ranged from 63.5 to 113.2% and were considered satisfactory, with relative standard deviations lower than 20%. The limits of quantification ranged from 0.14 to 6.73 μg kg^{-1}. The validated method was then applied to the determination of mycotoxins in 84 samples of cereal-based porridge destined for infant consumption obtained in the metropolitan region of Rio de Janeiro, RJ. Aflatoxins B1 and G1, fumonisins B1 and B2, hydrolyzed fumonisins B1 and B2, deoxynivalenol, and zearalenone were found, respectively, in 7.1, 2.4, 47.6, 3.6, 65.5, and 60.7% of the analyzed samples. The maximum permissible limits set by the Brazilian legislation were exceeded for at least one mycotoxin in 21 (25%) of the analyzed samples.

Keywords Mycotoxins · Infant food · Cereal-based porridge · UHPLC-MS/MS

Introduction

Mycotoxins are toxic compounds naturally produced as secondary metabolites by many filamentous fungi. Found as food contaminants worldwide, mycotoxins have caused significant economic losses in agriculture, and pose a very serious public health issue (Chu 1991; Murphy et al. 2006; Zain 2011; Rocha et al. 2014). In addition to several deleterious effects on human and animal health, some mycotoxins are classified by the International Agency for Research on Cancer (IARC) as human carcinogens or as potentially carcinogenic to humans (IARC 1993; Peraica et al. 1999; IARC 2002).

Taking into account their toxicity and occurrence, aflatoxins B1, B2, G1, and G2; deoxynivalenol; fumonisins B1 and B2; ochratoxin A; and zearalenone have been considered the main mycotoxins found in cereals and cereal-based products (Lee and Ryu 2017). Other mycotoxins have also been found in these foods, such as sterigmatocystin (Mol et al. 2016) and hydrolyzed fumonisins (Dombrink-Kurtzman and Dvorak 1999). Aflatoxins M1 and M2 and aflatoxins B1 and B2 hydroxylated metabolites, respectively, have been frequently found in milk (Peraica et al. 1999; Prandini et al. 2009; Sartori et al. 2015a). However, aflatoxins M1 and M2 may also be produced by fungi in minor amounts (Bräse et al. 2009; Filazi and Sireli 2013) and have also been reported in food other than milk, including corn (Shotwell et al. 1976; Vesonder et al. 1991; Ren et al. 2007; Huang et al. 2010; Ezekiel et al. 2012; Sartori et al. 2015b).

The contamination of cereal-based food destined for infant consumption by mycotoxins has been reported in several countries (Lombaert et al. 2003; Araguás et al. 2005; Tam

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et al. 2006; Baydar et al. 2007; D'Arco et al. 2008; Gottschalk et al. 2009; Kabak 2009; Alvito et al. 2010; Kostelanská et al. 2010; Romagnoli et al. 2010; Beltran et al. 2011; Cano-Sancho et al. 2011; Mahnine et al. 2012; Ozden et al. 2012; Rubert et al. 2012b; Juan et al. 2014; Zhang et al. 2014). In Brazil, high rates of mycotoxin occurrence in various cereals have been reported, including cereals used for the preparation of products intended for infant consumption (Maziero and Bersot 2010). However, few studies have determined mycotoxins in these products in Brazil (Castro et al. 2004; Caldas and Silva 2007).

Compliance with legislations regarding mycotoxin control in food requires the use of reliable analytical methods. In this scenario, several analytical approaches have been developed for the determination of mycotoxins in food (Krska et al. 2008; Cigić and Prosen 2009; Turner et al. 2009; Köppen et al. 2010; Saeger 2011). In recent years, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely applied in the determination of mycotoxins in different matrices (Turner et al. 2015; Berthiller et al. 2016; Berthiller et al. 2017). In addition, the increasing applicability of certain high-resolution mass spectrometry detectors for the determination of mycotoxins in food is also noted (Zachariasova et al. 2010; Rubert et al. 2012a; Fang et al. 2013; Jia et al. 2014; Righetti et al. 2016).

The selectivity of these techniques has enabled the simultaneous analysis of different classes of mycotoxins in several food matrices with minimum sample treatment (Sulyok et al. 2007; Mol et al. 2008; Frenich et al. 2009; Lacina et al. 2012). However, with regard to the determination of mycotoxins in food destined for infant consumption, most studies have reported more sophisticated sample treatment procedures using cleanup steps (Kostelanská et al. 2010; Rubert et al. 2012b; Zhang et al. 2014).

In this context, the aim of the present study was to develop and validate an analytical method for the determination of mycotoxins in commercial cereal-based porridge food destined for infant consumption by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Sample treatment involves a simultaneous extraction and cleanup (deffating) step, followed by concentration of the mycotoxin-containing extracts. The validated analytical method was then applied to the analysis of 84 samples of cereal-based porridge destined for infant consumption obtained from the metropolitan region of Rio de Janeiro, RJ.

Materials and Methods

Reagents and Chemicals

Acetonitrile and methanol (HPLC-grade) were purchased from J.T.Baker (Phillipsburg, NJ, USA). Ammonium formate (>99%) and formic acid (mass spectrometry grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *n*-Hexane (purity >96%), ethyl acetate (for analysis), and potassium hydroxide (pellets for analysis) were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

Standard Solutions

Solid standards of the aflatoxins B1, B2, G1, G2, M1, M2; ochratoxin A; and sterigmatocystin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of ochratoxin A (40 μg mL^{-1}) were prepared in toluene/acetic acid (99:1, *v*/*v*). Individual stock solutions of sterigmatocystin and of the aflatoxins were prepared in acetonitrile at 10 μg mL^{-1}. The concentrations of these standard solutions were determined by UV spectrophotometry (Horwitz and Latimer 2005). At least each 12 months, the stability of these solutions was checked by UV spectrophotometry. Stock solutions of fumonisins B1 and B2 (50 μg mL^{-1}) in acetonitrile/water (1:1, *v*/*v*) and deoxynivalenol in acetonitrile (100 μg mL^{-1}) were purchased from Fluka/Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of zearalenone in acetonitrile (100.7 μg mL^{-1}) were purchased from Biopure (Tulln, Austria). The hydrolyzed fumonisins B1 and B2 were prepared in the laboratory by hydrolysis of fumonisins B1 and B2 following the procedure described by Dall'Asta et al. (2009). Thus, an aliquot (5 mL) of a standard solution containing fumonisins B1 and B2 (50 μg mL^{-1}) in acetonitrile/water (1:1, *v*/*v*) was evaporated to dryness under a gentle nitrogen flow in a 40 °C water bath. The residues were dissolved in 5 mL of a 2 mol L^{-1} KOH solution and left to react for 12 h at room temperature. The hydrolyzed fumonisins were then extracted three times with 10 mL of ethyl acetate, combined, and evaporated to dryness under a gentle nitrogen flow in a 40 °C water bath, and the residues were subsequently dissolved in 5 mL of methanol. The absence of the native fumonisins in this solution was verified by UHPLC-MS/MS. Thus, total conversion to the hydrolyzed forms was assumed and the concentrations of hydrolyzed fumonisins B1 and B2 in methanol were calculated as 28.1 and 27.6 μg mL^{-1}, respectively. Aliquots from stock solutions were combined, and the volume was adjusted with methanol/water (1:1, *v*/*v*) to obtain an intermediate standard solution (10 ng mL^{-1} for ochratoxin A and aflatoxins M1, M2, G2, G1, B2, and B1; 40 ng mL^{-1} for sterigmatocystin; 200 ng mL^{-1} for hydrolyzed fumonisins B1 and B2, deoxynivalenol, and zearalenone; and 400 ng mL^{-1} for fumonisins B1 and B2). These solutions were diluted with methanol/water (1:1, *v*/*v*) to prepare working solutions. Intermediate and working solutions were prepared weekly. All the standard solutions were stored at −18 °C.

UHPLC-MS/MS Analysis

Liquid chromatography was performed using an ACQUITY UPLC™ system (Waters). A BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) was used as the stationary phase. The column temperature was maintained at 35 °C. The mobile phase flow rate was set at 0.3 mL min^{-1}. A 0.3% acid formic solution was used as the aqueous mobile phase of the elution gradient for the determination of fumonisins, hydrolyzed fumonisins, and sterigmatocystin. The elution gradient began with 60% methanol, was increased to 80% during 3 min, and was held at 80% for 1 min. The system was then re-equilibrated for 2 min with 60% methanol. The injection volume was of 5 μL. A 5 mmol L^{-1} formate ammonium solution was used as the aqueous mobile phase of the elution gradient used for the determination of aflatoxins M2, M1, B2, B1, G2, and G1; ochratoxin A; deoxynivalenol; and zearalenone. The elution gradient began with 10% methanol, was increased to 100% during 4 min, and was held at 100% for 1.5 min. The system was then re-equilibrated for 2 min with 10% methanol. The injection volume was of 7.5 μL.

Analyte detection was performed using a tandem quadrupole mass spectrometer (Waters, Quattro Premier™ XE) equipped with an electrospray ionization (ESI) source operated in both positive and negative ionization modes. The optimized source parameters were capillary voltage 3.5 kV, extractor voltage 3 V, rf lens 0.1 V, multiplier 750 V, desolvation temperature of 350 °C, and source temperature of 120 °C. Nitrogen was used as the cone and desolvation gas at flows of 50 and 750 L h^{-1}, respectively. Argon was used as the collision gas at a pressure of 4×10^{-3} mbar. The two ion transitions selected (*m/z*) for each mycotoxin and the acquisition conditions are displayed in Table 1.

Sample Preparation

Each sample (3 g) was weighed in 50-mL centrifuge tubes, and 5 mL of *n*-hexane, 5 mL of a 3% formic acid solution, and 10 mL of acetonitrile were added. The tubes were then shaken for 2 min using a vortex (IKA Works) and subsequently sonicated for 10 min. After these steps, the tubes were centrifuged at 3000 rpm for 7 min (Hitachi HIMAC CF 7D2). A 5-mL aliquot of the extracts (acetonitrile/water) was then concentrated to dryness under a gentle nitrogen flow in a 50 °C water bath (Turbo-Vac LV). Finally, the residues were dissolved with 1 mL of methanol/water (1:1, *v/v*) and the solutions thus obtained were filtered through 0.22-μm PVDF membrane filters.

Method Validation

Single laboratory validation was performed by evaluating the following analytical performance parameters: selectivity, matrix effect, linearity, trueness, precision (repeatability and intermediate precision), limit of detection (LOD), and limit of quantification (LOQ).

The selectivity of the method was evaluated by analyzing matrix blank samples (corn-based, oat-based, rice-based, oat/rice-based, and multicereal-based) regarding the presence of

Table 1 UHPLC-MS/MS parameters for the target mycotoxins

Mycotoxins	t_R (min)	Quantifier transition ion, Q (*m/z*)	Qualifier transition ion, q (*m/z*)	Cone voltage (V)	Energy collision (eV)[a]	Q/q[b]
Hydrolyzed fumonisin B1	1.60	406.3 > 388.3	406.3 > 370.3	30	20/20	1.2 (±0.2)
Fumonisin B1	1.75	722.2 > 334.3	722.2 > 352.3	50	40/40	1.2 (±0.2)
Hydrolyzed fumonisin B2	2.47	390.3 > 372.3	390.3 > 354.3	30	20/20	1.3 (±0.3)
Fumonisin B2	2.72	706.2 > 336.3	706.2 > 318.3	50	35/35	2.0 (±0.4)
Sterigmatocystin	3.00	325.2 > 281.2	325.2 > 310.2	45	35/25	1.1 (±0.2)
Deoxynivalenol	2.15	297.1 > 249.1	297.1 > 231.1	25	25/25	2.2 (±0.6)
Aflatoxin M2	2.91	331.3 > 273.3	331.3 > 285.2	45	25/25	1.9 (±0.4)
Aflatoxin G2	3.04	331.3 > 245.3	331.3 > 285.3	40	30/30	1.5 (±0.3)
Aflatoxin M1	3.05	329.2 > 273.2	329.2 > 259.2	50	25/25	2.1 (±0.5)
Aflatoxin G1	3.16	329.2 > 243.2	329.2 > 283.2	45	25/25	1.5 (±0.3)
Aflatoxin B2	3.28	315.2 > 287.0	315.2 > 259.2	50	25/30	1.0 (±0.2)
Aflatoxin B1	3.38	313.0 > 269.2	313.0 > 285.2	40	35/25	1.8 (±0.4)
Ochratoxin A	3.75	404.2 > 239.2	404.2 > 358.2	25	25/15	1.6 (±0.3)
Zearalenone	4.23	316.9 > 174.8	316.9 > 130.8	50	25/30	1.3 (±0.3)

ESI in positive mode for all analytes, except for zearalenone

[a] Values are given in the order quantifier transition ion/qualifier transition ion

[b] Relative ion transition intensities (Q/q) and maximum permitted tolerances given in parentheses (EC 2002)

interfering signals eluted at the same time as the analytes in all porridge samples. The mycotoxins were identified in the samples by comparing the analyte retention times to the standard solution retention times. Confirmation was performed by comparison of the signal intensity ratios of the quantifier and qualifier ion transitions of each analyte in the samples with those obtained using standard solutions considering the maximum permitted tolerances according to the European Union (EC 2002).

To investigate matrix effects, calibration curves for each compound in the matrix extract (matrix-matched calibration) and in methanol/water (1:1, *v/v*) were prepared at eight concentration levels, ranging from 0.25 to 5 ng mL^{-1} (aflatoxins M2, M1, G2, G1, B2, and B1, and ochratoxin A), 1 to 20 ng mL^{-1} (sterigmatocystin), 5 to 100 ng mL^{-1} (hydrolyzed fumonisins B1 and B2, deoxynivalenol, and zearalenone), and 10 to 200 ng mL^{-1} (fumonisins B1 and B2). The calibration curve slopes were compared by an analysis of covariance (ANCOVA), considering a significance level of 5% (García-Campaña et al. 1997). Before the ANCOVA analysis, the homogeneity of the residual variances of all the calibration curves was verified (p values >0.05) applying a modified Levene test (Brown and Forsythe 1974).

Linearity was assessed using calibration curves with the same concentration levels used when studying the matrix effect (Souza and Junqueira 2005). Outliers were successively investigated by the Jackknife standardized residuals test (Belsley et al. 1980). The homoscedasticity of the residuals for all the calibration curves was verified by a modified Levene test (Brown and Forsythe 1974). The independency of the residuals for all calibration curves was verified by a Durbin-Watson statistical test (Durbin and Watson 1951). The normality of the residuals for all the calibration curves was verified by Ryan-Joiner's test (Ryan and Joiner 1976). The regression significance and the lack of fit were performed by an analysis of variance (ANOVA) (Draper and Smith 1998).

The trueness and repeatability of the method were evaluated by recovery studies using oat/rice-based porridge samples spiked with the mycotoxins at four concentration levels, with four replicates for each level. Intermediate precision was performed by the analysis of spiked samples with the same concentrations used in the second concentration level, analyzed within 3 days by three different analysts.

Porridge samples spiked with the compounds in the lowest concentration level used in the recovery studies were used to determine the limit of detection (LOD) and limit of quantification (LOQ), considering signal-to-noise ratios of 3 and 10, respectively.

Samples of Cereal-Based Porridge Destined for Infant Consumption

A total of 84 samples of commercial cereal-based porridge destined for infant consumption were purchased randomly from local supermarkets in the metropolitan region of Rio de Janeiro, RJ, Brazil, between 2012 and 2014, from 13 different companies, representing 37 different brands, classified as corn-based (n = 30), rice-based (n = 20), oat-based (n = 7), oat/rice-based (n = 10), wheat-based (n = 3), and multicereal-based (n = 14). The samples were stored in their original packaging at −20 °C until analysis.

Results and Discussion

Optimization of the UHPLC-MS/MS Conditions

The mass spectrometry conditions were optimized by infusion of individual working standard solutions, between 500 and 1000 ng mL^{-1}, using an *ESI* source operated in both positive and negative ionization modes. Initially, more intense precursor ions were selected and the cone voltage was optimized for each target compound with the mass spectrometer operating in the scan mode. Subsequently, collision energies were applied to obtain the product ions. Collision energies were optimized for each transition, and the two more intense product ions were selected as the quantification and confirmation transition ions. Ion transitions obtained in positive mode were selected for all mycotoxins, except for zearalenone. For this mycotoxin, the transitions obtained in positive ionization mode (m/z 319.3 > 301.2 and m/z 319.3 > 283.2) showed interfering peaks when the sample preparation method was applied to the cereal-based porridge samples. Thus, the ion transitions obtained in the negative mode were selected for zearalenone, providing satisfactory method sensitivity and selectivity (Fig. 1).

The mobile phase composition was studied with the purpose of obtaining adequate chromatographic peak shapes and sensitivity for the mycotoxin analysis. The presence of *carryover* was evaluated by injecting a solution of target mycotoxins followed by solvent injections (methanol/water (1:1, *v/v*)).

Methanol was selected as the organic mobile phase because it provides higher sensitivity when compared to acetonitrile. Different compositions of the initial organic mobile phase (10, 25, 55, and 60%), with a linear gradient ending at 80 and 90% of the organic mobile phase, were evaluated. The efficiency of various additives in both mobile phases or only in the aqueous mobile phase (ammonium formate 5 mmol L^{-1}, 0.1% of acid formic, 0.3% of acid formic, ammonium formate 5 mmol L^{-1}/0.1% of acid formic, ammonium formate 5 mmol L^{-1}/0.3% of acid formic), added to provide ionization and adequate peak shape for the analytes, was also studied.

A decrease in the concentration of the organic solvent in the initial mobile phase was found to increase sensitivity to the target compounds; adequate sensitivity was observed by using 10 and 25% of organic phase in the initial gradient; *carryover* was negligible (<1%) for ochratoxin A using these methods.

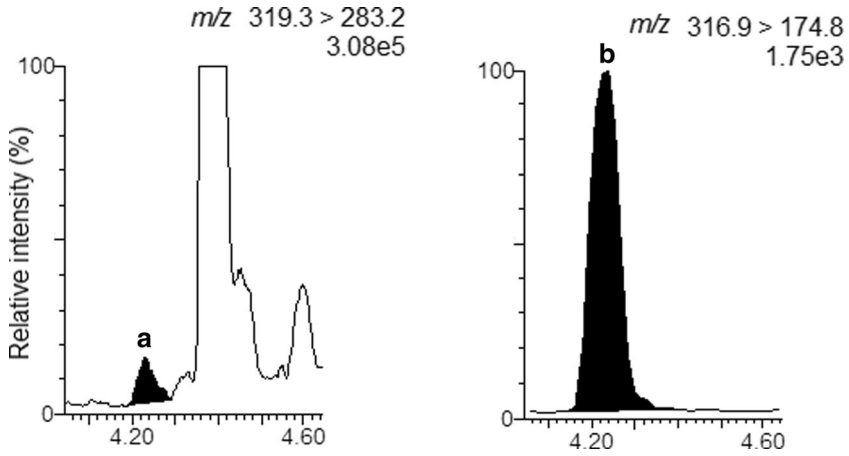

Fig. 1 Chromatograms of a corn/oat-based porridge sample fortified with the zearalenone (10 μg kg^{-1}) indicating **a** presence of matrix interferents (*m/z* 319.3 > 283.2) and **b** absence of matrix interferents (*m/z* 316.9 > 174.8)

For sterigmatocystin, a significant *carryover* (>3%) was observed. As reported previously (Plattner 1999; Tamura et al. 2012), the worst *carryovers* were observed for the fumonisins, mainly for fumonisin B2, since this compound appeared in several injection cycles after the injection of a standard solution or contaminated sample. In order to solve this problem, several solvents (methanol; 0.1% formic acid in methanol; 0.3% formic acid in methanol; 1% formic acid in methanol; methanol/water (1:1, *v/v*); 1% formic acid in water, acetonitrile, methanol (1:1:1:1, *v/v/v*)) were tested to be used as the injection syringe wash solvents. The tested washing solvents were not efficient in eliminating the *carryover* (fumonisins continued to appear in several injection cycles). This effect occurs due to the possible presence of reversible bonds of these substances with metal ions in the sample path of the chromatographic system, including the chromatographic columns (Tamura et al. 2014). Decrease of *carryover* was observed with increasing ratios of organic solvents in the initial mobile phase gradient, as previously reported (Tamura et al. 2011). In our study, absence of *carryover* was observed for fumonisins and sterigmatocystin using 60% of the organic solvent in the initial mobile phase gradient, 0.3% formic acid as the aqueous mobile phase, and a linear gradient ending in 80% of organic phase. As these conditions are inadequate for the determination of all the target compounds using one gradient, two methods were selected: the conditions described above were used for the determination of the fumonisins, hydrolyzed fumonisins, and sterigmatocystin, while 10% of organic solvent (methanol) in the initial gradient and a 5-mmol L^{-1} ammonium formate solution as the aqueous mobile phase were selected for aflatoxins M2, M1, B2, B1, G2, and G1; ochratoxin A; deoxynivalenol; and zearalenone.

After the selection of the mobile phase, different injection volumes (5, 7.5, and 10 μL) were evaluated with regard to chromatographic peak shape and method sensitivity. An injection volume of 5 μL was selected for fumonisins, hydrolyzed fumonisins, and sterigmatocystin, due to adequate sensitivity for all target substances. An injection volume of 7.5 μL was chosen for the determination of the other mycotoxins, since this is the highest volume that did not lead to chromatographic problems, mainly for deoxynivalenol.

Optimization of the Sample Treatment Method

Four methods frequently reported in the literature for the simultaneous determination of different classes of mycotoxins in foods were initially selected for evaluation (Sulyok et al. 2007; Mol et al. 2008; Tamura et al. 2011; Lacina et al. 2012). These methods were modified in order to reduce the analysis time and improve sensitivity. The experiments were performed in triplicate using oat/rice-based porridge samples fortified with target mycotoxins. Recoveries were determined using blank extracts fortified with mycotoxins at adequate concentrations, avoiding the influence of the matrix effect. The evaluated methods are described as follows. Method A, modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Tamura et al. 2011): 10 mL of water and 10 mL of acetonitrile were added to 3 g of the sample weighed in a 50-mL centrifuge tube. The mixture of salts (QuEChERS citrate) was then added, and the tube was immediately shaken for 1 min and centrifuged at 3000 rpm for 7 min. A 3-mL aliquot of the acetonitrile phase was evaporated to dryness under a gentle nitrogen flow at 45 °C. The residue was then dissolved with 1 mL of methanol/water (1:1, *v/v*). The solution thus obtained was filtered through a 0.22-μm filter before injection. Method B, modified QuEChERS method (Lacina et al. 2012): 10 mL of formic acid solution 2% and 10 mL of acetonitrile were added to 3 g of the sample weighed in a 50-mL centrifuge tube. A mixture of salts (4 g of magnesium sulfate and 1 g of sodium chloride) was then added, and the tube was immediately shaken for 1 min and centrifuged at 3000 rpm for 7 min. A 3-mL aliquot of the acetonitrile phase was evaporated to dryness under a gentle nitrogen flow at 45 °C. The residue was then dissolved with 1 mL of methanol/water (1:1, *v/v*). The solution thus obtained was filtered through a 0.22-μm filter before injection. Method C,

Table 2 Recoveries (%) obtained for the evaluated methods

Mycotoxins	Method A (n = 3)	Method B (n = 3)	Method C (n = 3)	Method D (n = 3)
Deoxynivalenol	72.9 (6.4)	85.8 (1.3)	64.9 (4.3)	80.5 (1.5)
Aflatoxin M2	90.0 (5.1)	86.3 (3.9)	76.8 (28.8)	95.2 (6.8)
Aflatoxin M1	87.8 (7.4)	87.2 (3.2)	79.5 (5.9)	93.6 (9.3)
Aflatoxin G2	87.6 (2.0)	97.2 (1.0)	80.8 (3.7)	87.4 (2.3)
Aflatoxin G1	47.7 (13.3)	91.5 (2.0)	74.0 (2.0)	89.2 (7.9)
Aflatoxin B2	85.8 (4.7)	95.4 (2.6)	68.7 (6.3)	89.3 (7.2)
Aflatoxin B1	46.9 (11.1)	95.6 (0.4)	64.6 (4.3)	83.8 (8.3)
Fumonisin B1	63.4 (14.8)	50.8 (8.4)	52.5 (14.2)	114.7 (2.0)
Fumonisin B2	70.0 (12.9)	43.9 (12.8)	43.9 (11.2)	107.5 (4.2)
Hydrol. Fum. B1	21.0 (29.3)	24.2 (21.8)	30.3 (9.4)	49.9 (2.3)
Hydrol. Fum. B2	12.7 (29.7)	14.7 (23.2)	23.8 (9.9)	29.8 (0.7)
Ochratoxin A	68.2 (0.5)	89.1 (4.9)	55.7 (6.1)	107.3 (3.9)
Zearalenone	78.9 (4.7)	103.1 (1.0)	63.6 (9.1)	86.1 (8.0)
Sterigmatocystin	36.3 (11.4)	80.1 (1.6)	52.0 (5.0)	94.9 (2.6)

RSD (%) given in parentheses; spiked concentration level: 2.5 μg kg^{-1} for aflatoxins M1 and M2, 10 μg kg^{-1} for aflatoxins G2, G1, B2, and B1, ochratoxin A, and sterigmatocystin; 25 μg kg^{-1} for hydrolyzed fumonisins B1 and B2; 50 μg kg^{-1} for deoxynivalenol, zearalenone, and fumonisins B1 and B2

solid-liquid extraction (Sulyok et al. 2007): 5 mL of formic acid solution 5% was added to 3 g of the sample weighed in a 50-mL centrifuge tube. Subsequently, 20 mL of acetonitrile was added and the tube was shaken for 2 min and sonicated for 10 min. The tube was then centrifuged at 3000 rpm for 7 min. A 5-mL aliquot of the extract was evaporated to dryness under a gentle nitrogen flow at 45 °C. The residue was then dissolved with 1 mL of methanol/water (1:1, *v*/*v*). The solution thus obtained was filtered through a 0.22-μm filter before injection. Sonication was used instead of shaking (60 min) used in the original methods. Method D, solid-liquid extraction (Mol et al. 2008): 5 mL of water was added to 3 g of the sample weighed in a 50-mL centrifuge tube. Subsequently, 15 mL of acetonitrile (1% formic acid) was added and the tube was shaken for 2 min and then sonicated for 10 min. The tube was then centrifuged at 3000 rpm for 7 min. A 5-mL aliquot of the extract was evaporated to dryness under a gentle nitrogen flow at 45 °C. The residue was then dissolved with 1 mL of methanol/water (1:1, *v*/*v*). The solution thus obtained was filtered through a 0.22-μm filter before injection. Sonication was used instead of shaking (60 min) used in the original methods.

Table 3 Conditions evaluated in the method optimization for determination of the mycotoxins

Experiment	Hexane (mL)	5 mL of water or 3% formic acid	Acetonitrile (mL)	Sonication (min)
1	0	Water	10	10
2	0	Water	15	10
3	0	formic acid 3%	10	10
4	0	formic acid 3%	15	10
5	5	Water	10	10
6	5	Water	15	10
7	5	formic acid 3%	10	10
8	5	formic acid 3%	15	10
9	10	Water	10	10
10	10	Water	15	10
11	10	formic acid 3%	10	10
12	10	formic acid 3%	15	10
13	5	formic acid 3%	10	30

The obtained results are displayed in Table 2. None of the evaluated methods obtained satisfactory results (70 to 120%) for all the evaluated mycotoxins, although satisfactory recoveries were obtained using method D for all mycotoxins except for hydrolyzed fumonisins B1 (49.9%) and B2 (29.8%). However, a better extraction efficiency of hydrolyzed fumonisins was observed in this method compared to method C (hydrolyzed fumonisins B1 (30.3%) and B2 (23.8%), demonstrating a possible relationship of extraction efficiency with the water/acetonitrile ratio used in these methods.

Taking into account these results, several experiments were conducted combining different extraction solvents and sonication times, in order to obtain an efficient method for the extraction of the target mycotoxins, including hydrolyzed fumonisins. Oat/rice-based porridge samples fortified with target mycotoxins were used in the experiments. A total of 13 combined experiments were performed in duplicate, described as follows: 5 mL of *n*-hexane was added (or not added) to 3 g of the sample weighed in a 50-mL centrifuge tube. Subsequently,

Table 4 Recovery results (%) obtained for the method optimization

Exp.[a]	DON	AFM2	AFM1	AFG2	AFG1	AFB2	AFB1	FB1	FB2	HFB1	HFB2	OTA	ZEA	STG
1	109.6 (13.6)	111.8 (16.1)	98.8 (8.4)	102.1 (4.8)	112.3 (1.8)	108.1 (3.8)	109.6 (3.4)	45.2 (9.9)	58.7 (12.5)	45.9 (4.4)	35.2 (12.5)	89.8 (14.4)	113.5 (11.7)	101.8 (4.8)
2	89.9 (2.2)	102.2 (2.2)	82.1 (13.8)	96.4 (6.2)	100.6 (4.4)	92.1 (4.2)	89.9 (6.6)	10.4 (4.4)	14.8 (8.7)	30.1 (4.7)	18.7 (0.9)	109.5 (9.9)	92.0 (2.0)	88.1 (2.6)
3	94.4 (5.9)	100.7 (4.0)	105.4 (14.8)	106.7 (7.8)	100.4 (4.4)	104.1 (9.5)	101.2 (6.7)	105.0 (4.9)	108.6 (8.8)	57.7 (8.1)	44.4 (11.2)	96.7 (15.4)	107.6 (20.6)	98.8 (3.5)
4	80.5 (1.5)	95.2 (6.8)	93.6 (9.3)	87.4 (2.3)	89.2 (7.9)	89.3 (7.2)	83.8 (8.3)	114.7 (2.0)	107.5 (4.2)	49.9 (2.3)	29.8 (0.7)	107.3 (3.9)	86.1 (8.0)	94.9 (2.6)
5	98.1 (0.7)	119.5 (1.2)	112.8 (1.3)	122.3 (0.2)	122.9 (2.7)	116.4 (3.6)	121.2 (0.1)	21.3 (1.3)	31.9 (1.6)	60.4 (1.6)	41.5 (1.6)	128.2 (4.2)	124.7 (4.1)	117.7 (2.4)
6	93.3 (5.2)	115.3 (3.8)	141.6 (14.0)	117.9 (15.2)	108.2 (5.6)	109.5 (5.2)	107.0 (3.7)	4.9 (5.3)	9.4 (7.6)	47.2 (1.6)	33.6 (1.9)	110.3 (10.4)	105.6 (2.5)	99.0 (4.6)
7	84.1 (2.8)	104.9 (31.4)	111.6 (20.8)	107.4 (1.7)	108.7 (4.1)	113.1 (2.8)	102.4 (8.7)	95.2 (9.2)	99.3 (12.6)	71.9 (8.3)	63.0 (12.9)	103.9 (14.4)	103.6 (13.2)	91.1 (6.7)
8	87.4 (4.5)	102.1 (1.5)	97.9 (0.9)	118 (0.4)	106.2 (0.9)	105.1 (0.7)	94.6 (0.2)	95.7 (2.2)	105.7 (5.5)	61.0 (3.7)	46.5 (1.0)	107.4 (7.2)	84.3 (5.5)	82.5 (2.8)
9	92.8 (6.2)	103.3 (4.2)	108.5 (9.5)	110.0 (4.6)	119.0 (3.2)	113.2 (5.7)	110.5 (3.0)	27.6 (9.7)	58.7 (31.0)	56.9 (3.3)	40.0 (1.8)	110.9 (6.6)	113.0 (6.6)	108.3 (2.6)
10	95.2 (8.8)	119.8 (8.5)	124.8 (24.1)	113.1 (8.9)	114.5 (4.7)	111.1 (4.8)	110.3 (1.6)	5.3 (6.6)	10.1 (20.2)	44.7 (2.9)	34.0 (3.0)	130.8 (4.5)	105.2 (2.8)	111.7 (2.8)
11	100.3 (2.9)	111.9 (2.1)	122.3 (1.7)	113.4 (4.6)	109.3 (4.3)	117.9 (4.2)	101.3 (6.0)	106.3 (6.0)	102.1 (3.9)	77.0 (2.4)	61.9 (5.7)	77.9 (7.9)	127.4 (5.6)	92.8 (3.0)
12	59.4 (17.2)	78.2 (20.0)	81.4 (14.1)	80.0 (17.9)	69.5 (12.5)	71.1 (17.2)	69.4 (14.6)	70.9 (41.3)	72.5 (36.7)	42.9 (8.6)	37.3 (11.9)	114.5 (10.8)	77.9 (10.5)	88.4 (3.2)
13	93.6 (4.2)	104.1 (7.4)	112.4 (8.1)	100.9 (10.5)	99.1 (3.8)	101.5 (4.7)	96.1 (6.7)	112.6 (5.9)	116.9 (7.2)	73.2 (1.2)	57.1 (4.3)	106.5 (15.7)	103.5 (8.3)	94.3 (3.6)

RSD (%) is given in parentheses. Spiked concentration level: 2.5 μg kg^{-1} for aflatoxins M1 and M2; 10 μg kg^{-1} for aflatoxins G2, G1, B2, and B1, ochratoxin A, and sterigmatocystin; 25 μg kg^{-1} for hydrolyzed fumonisins B1 and B2; and 50 μg kg^{-1} for deoxynivalenol, zearalenone, and fumonisins B1 and B2

DON deoxynivalenol, *AFM2* aflatoxin M2, *AFM1* aflatoxin M1, *AFG2* aflatoxin G2, *AFG1* aflatoxin G1, *AFB2* aflatoxin B2, *AFB1* aflatoxin B1, *FB1* fumonisin B1, *FB2* fumonisin B2, *HFB1* hydrolyzed fumonisin B1, *HFB2* hydrolyzed fumonisin B2, *OTA* ochratoxin A, *ZEA* zearalenone, *STG* sterigmatocystin

[a] Experimental conditions are displayed in Table 3

5 mL of water (or formic acid 3% solution) and 10 mL (or 15 mL) of acetonitrile were added. The tube was shaken for 2 min using a vortex and then sonicated for 10 min (or 30 min). The sample was then centrifuged at 3000 rpm for 7 min. A 5-mL aliquot of the acetonitrile/water phase was evaporated to dryness under a gentle nitrogen flow at 50 °C (Turbo-Vac LV). The residue was then dissolved with 1 mL of methanol/water (1:1, *v*/*v*). The solution thus obtained was filtered through a 0.22-μm filter. The conditions of each experiment and their results are displayed in Tables 3 and 4, respectively. The best results for target mycotoxins were obtained, including hydrolyzed fumonisins, using *n*-hexane (5 and 10 mL), a 3% formic acid solution, and 10 mL of acetonitrile in the extraction procedure. No significant difference was observed in the extraction efficiency for hydrolyzed fumonisins using 5 or 10 mL of *n*-hexane; since it uses less amount of solvent, the experiment condition using 5 mL of *n-hexane* was selected to investigate the influence of sonication time on the extraction of hydrolyzed fumonisins. However, the extraction efficiency of hydrolyzed fumonisins was not improved with increased extraction time (30 min). Thus, the procedure using 5 mL of *n*-hexane, a 3% formic acid solution, 10 mL of acetonitrile, and sonication for 10 min during the extraction step was selected for validation.

Method Validation

The developed analytical method was validated for the determination of 14 mycotoxins (aflatoxins M2, M1, G2, G1, B2, B1; deoxynivalenol; ochratoxin A; fumonisins B1 and B2; hydrolyzed fumonisins B1 and B2; zearalenone; and sterigmatocystin) in cereal-based porridge destined for infant consumption.

No interfering signals eluted at the same time as the analytes in all porridge samples (corn-based, oat-based, rice-based, oat/rice-based, and multicereal-based). Figure 2 displays a chromatogram of an oat/rice-based porridge sample fortified with target mycotoxins at the limit of quantification. The retention times and ion ratios, as well as the maximum permitted tolerances for ion ratios obtained for the target mycotoxins, are displayed in Table 1.

The wide variety of porridge brands available in the market, as well as the absence of uncontaminated matrices for some mycotoxins, made it difficult to carry out matrix effect studies for all brands. Therefore, to evaluate the extension of the phenomena, matrix effects were studied using only the oat/rice-based porridge matrix.

Significant differences between the slopes of the calibration curves prepared in the solvent and in the matrix for several target mycotoxins were observed (p values <0.05), indicating that the matrix effect is significant for these compounds.

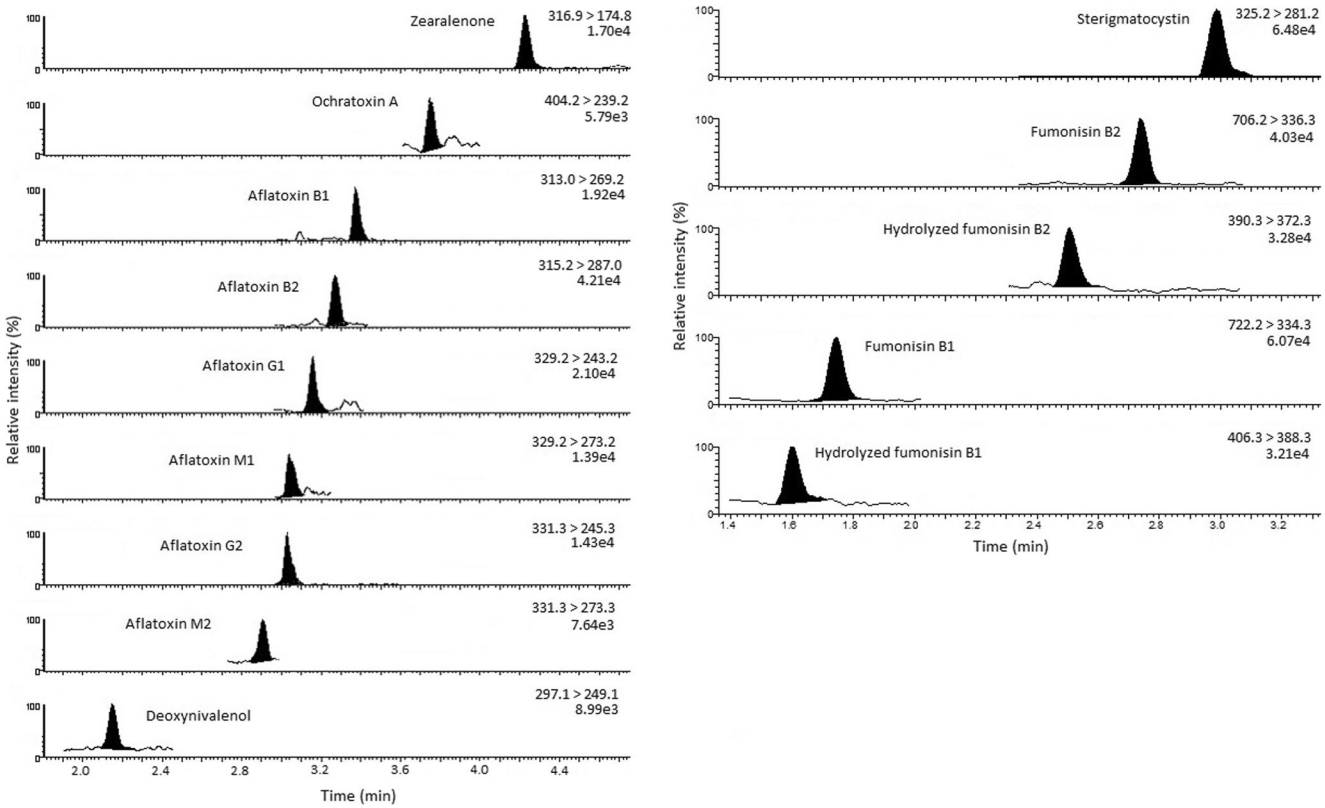

Fig. 2 Chromatograms of an oat/rice-based porridge sample fortified with the target mycotoxins at the limit of quantification

Table 5 Matrix effects (%) for target mycotoxins in different extract dilutions (oat/rice-based porridge)

Mycotoxins	1.0 g mL^{-1}	0.5 g mL^{-1}	0.25 g mL^{-1}	0.1 g mL^{-1}
Deoxynivalenol	−22	−19	−15	−11
Aflatoxin M2	5	25	22	13
Aflatoxin M1	−23	−9	−10	−20
Aflatoxin G2	−72	−64	−59	−42
Aflatoxin G1	−58	−47	−36	−21
Aflatoxin B2	−57	−36	−24	−12
Aflatoxin B1	−56	−37	−24	−15
Fumonisin B1	29	19	9	18
Fumonisin B2	33	22	6	20
Hydrolyzed fumonisin B1	34	27	6	21
Hydrolyzed fumonisin B2	31	29	5	26
Ochratoxin A	2	38	50	46
Zearalenone	−43	−21	−7	5
Sterigmatocystin	−4	6	−5	5

Sample dilutions (final extracts) and chromatographic gradient time effects were thus studied in order to reduce or eliminate matrix effects. The effect of sample dilution was assessed using four matrix proportions in the final extract (0.1, 0.25, 0.5, and 1 g mL^{-1}), with the same mycotoxin concentrations in all extracts. The obtained results are displayed in Table 5. A decrease in the matrix effect for most mycotoxins was observed when increasing sample dilution. However, a significant matrix effect (>10%) for most mycotoxins was observed for all evaluated dilutions.

In an attempt to decrease the matrix effect, the gradient time was increased from 4 to 6 min. However, even though the matrix effect decreased up to 49% when increasing the gradient time for most of the assessed mycotoxins, a gradient time of 4 min was applied because of losses of chromatographic signal intensities (20 to 65%) and increases in the analysis time. Thus, matrix-matched calibration was used in routine analysis, while the standard addition method was used for quantifying mycotoxins when concentrations were higher than the maximum permissible concentration and when an uncontaminated matrix was not available to prepare matrix-matched calibrations (Mavungu et al. 2009; EC 2014).

In the linearity studies, the homoscedasticity, the independency of the residuals, and the normality of the residuals for all the calibration curves were confirmed (p values >0.05). A high regression significance (p values <0.001) and non-significant lack of fit (p values >0.05) were found, attesting to the linearity of the evaluated curves.

The trueness and repeatability results of the method are displayed in Table 6, where the repeatability and intermediate precision are expressed by the relative standard deviation (RSD %) and trueness by the recovery values. The recovery values ranged from 63.5 to 113.2%, with RSD lower than 20% for all studied mycotoxins under repeatability conditions. The RSD for the intermediate precision study was always lower than 12%. The results were satisfactory according the European Union criteria (EC 2002; EC 2006).

Table 6 Validation parameters of the selected method

			1 μg kg^{-1a}		5 μg kg^{-1a}			10 μg kg^{-1a}		20 μg kg^{-1a}	
Mycotoxins	LOD	LOQ	Rec	RSD_r	Rec	RSD_r	RSD_R	Rec	RSD_r	Rec	RSD_r
Deoxynivalenol	2.02	6.73	99.5	3.1	83.9	12.2	4.7	94.5	20.0	63.5	8.6
Aflatoxin M2	0.11	0.38	86.5	9.6	88.0	9.4	5.7	106.8	16.8	79.5	5.3
Aflatoxin M1	0.08	0.27	88.3	12.9	92.6	9.4	2.1	110.6	19.9	73.0	9.2
Aflatoxin G2	0.25	0.82	75.3	10.5	81.8	6.9	8.2	109.2	17.0	84.6	5.5
Aflatoxin G1	0.16	0.53	83.1	9.7	90.5	9.1	4.3	99.2	13.4	79.5	4.3
Aflatoxin B2	0.14	0.46	110.4	11.2	96.9	5.2	6.0	112.0	18.1	89.4	3.9
Aflatoxin B1	0.07	0.23	90.0	8.1	83.3	7.1	2.8	94.2	17.7	82.4	3.9
Fumonisin B1	0.54	1.81	83.8	5.9	88.4	16.8	9.6	107.5	7.5	103.0	5.3
Fumonisin B2	0.45	1.51	81.9	8.8	85.2	11.8	8.5	113.2	8.5	102.4	3.0
Hydrolyzed fumonisin B1	0.95	3.18	93.2	9.5	95.3	5.4	11.6	89.4	7.1	82.5	6.3
Hydrolyzed Fumonisin B2	0.92	3.07	91.8	14.9	87.8	4.1	9.0	84.6	7.2	76.0	7.3
Ochratoxin A	0.13	0.43	92.3	8.4	99.0	11.8	5.0	106.5	10.7	103.4	5.5
Zearalenone	0.64	2.12	112.3	5.3	85.9	11.0	7.7	97.0	9.8	101.2	0.6
Sterigmatocystin	0.04	0.14	102.2	8.7	91.8	3.2	2.4	99.9	5.7	94.5	2.7

LOD limit of detection (μg kg^{-1}), *LOQ* limit of quantification (μg kg^{-1}), *Rec* recovery (%), RSD_r (%) relative standard deviation (intra-day, $n = 4$), RSD_R (%) relative standard deviation (inter-day, $n = 3$)

[a] Spiked concentration levels for aflatoxins G2, G1, B2, and B1; ochratoxin A; and sterigmatocystin (half of the concentrations for aflatoxins M2 and M1 and ten times larger for deoxynivalenol, zearalenone, fumonisins B1 and B2, and hydrolyzed fumonisins B1 and B2) in oat/rice-based porridge samples

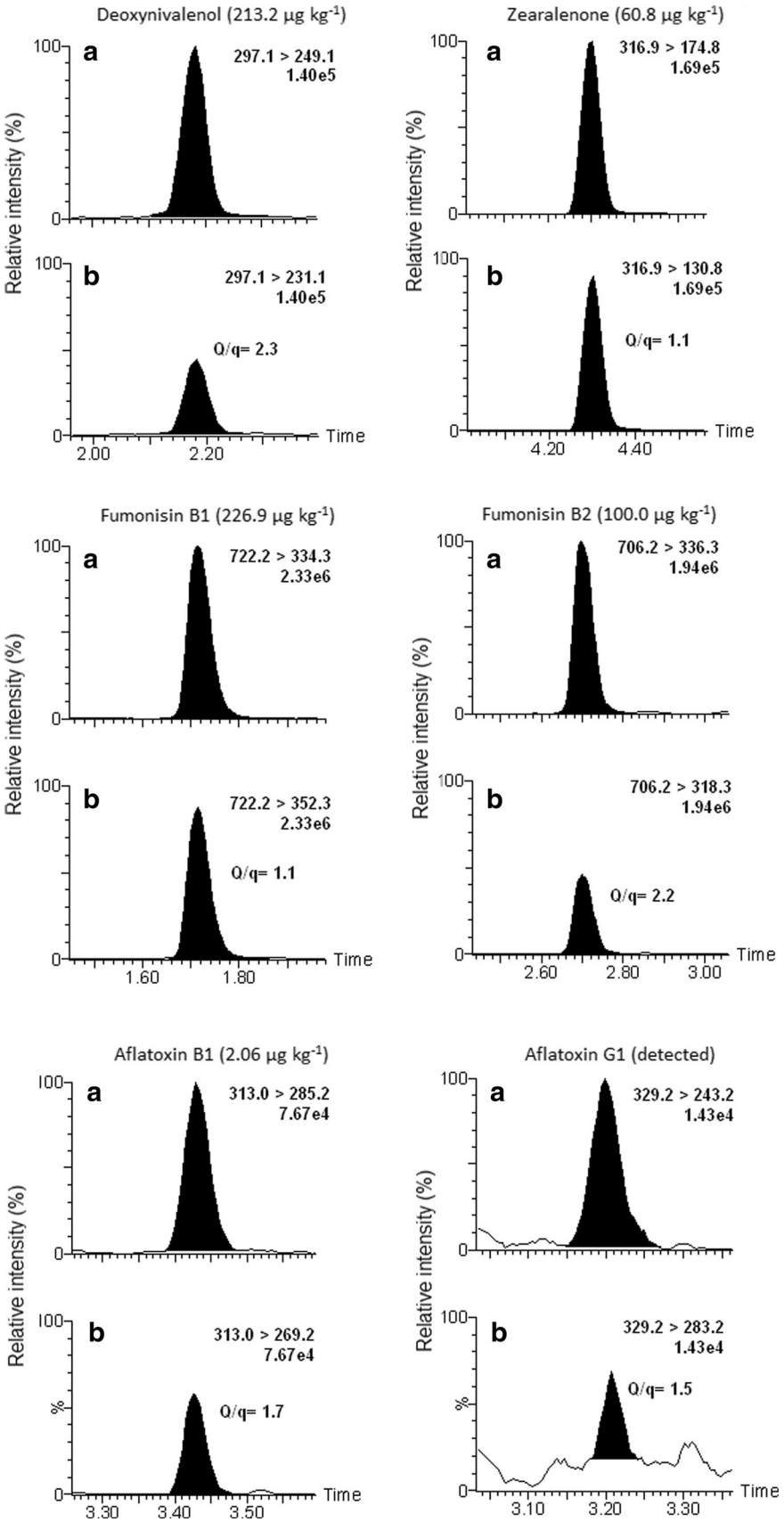

Fig. 3 Chromatograms of cereal-based porridge samples naturally contaminated by mycotoxins

The values obtained for LOD and LOQ are also displayed in Table 6. The sensitivity of the method was considered suitable for the routine analysis of target mycotoxins in cereal-based porridge destined for infants, taking into account the maximum limit allowed for regulated mycotoxins (FAO 2004; Brasil 2011).

Sample Analyses

After validation, the developed method was used to determine target mycotoxins in samples of cereal-based porridge intended for infant consumption. The results are displayed in supplementary material (Online Resource 1).

The results were evaluated according to the normative resolution RDC No. 07/2011, of the current Brazilian legislation for food mycotoxin control (Brasil 2011). The permissible limits set by the Brazilian legislation were exceeded for at least one of the investigated mycotoxins in 21 (25%) of the samples.

Aflatoxin B1 was found in 6 (7.1%) of the analyzed samples at concentration levels between 0.07 and 2.06 μg kg^{-1}. The incidence of aflatoxin B1 in the present study is low when compared to the 88, 50, and 25% values found by Baydar et al. (2007), Tam et al. (2006), and Alvito et al. (2010), respectively, when analyzing cereal-based foodstuffs for infant consumption.

Aflatoxin B1 exceeded the maximum limit allowed for the sum of aflatoxins B1, B2, G1, and G2 (1 μg kg^{-1}) in cereal-based foodstuffs for infant consumption set by the Brazilian legislation in one sample (corn-based porridge). Aflatoxin G1 was detected in 2 (2.4%) of the samples.

Fumonisins B1 and B2 were both found in 40 (47.6%) of the analyzed samples. Fumonisins (B1 + B2) were found in concentration levels ranging 7 from 1500 μg kg^{-1}, with an average of 283 μg kg^{-1}; the concentration levels found in 15 (18%) of the analyzed samples exceeded the maximum permissible limit (200 μg kg^{-1}) according to the Brazilian legislation.

These mycotoxins were found in all (100%) of the corn-based porridge samples. The high incidence and levels of fumonisins found in the corn-based porridge samples are in agreement with previous reports for corn-based foods destined for infant consumption in Brazil (Castro et al. 2004).

The hydrolyzed fumonisins B1 and B2 were found in 3 (3.6%) of the analyzed samples, indicating that fumonisins can be hydrolyzed during the industrial processing of these foods (Dombrink-Kurtzman and Dvorak 1999).

Deoxynivalenol was found in 55 (65.5%) of the analyzed samples at concentrations ranging from 2.0 to 332.5 μg kg^{-1}, with an average of 68.1 μg kg^{-1}. Concentrations exceeded the maximum limit (200 μg kg^{-1}) set by the Brazilian legislation in 5 (6.0%) of the analyzed samples. The incidence of deoxynivalenol found herein was lower than that reported by Juan et al. (2014), of 76%, and higher than the 63, 40, 36, and 23.5% reported by Lombaert et al. (2003), Cano-Sancho et al. (2011), Romagnoli et al. (2010), and Zhang et al. (2014), respectively, in cereal-based foods for infant consumption.

Zearalenone was found in 51 (60.7%) of the analyzed samples at concentrations ranging between 0.64 and 60.8 μg kg^{-1}, averaging 6.7 μg kg^{-1}. The concentrations exceeded the maximum permissible levels allowed (20 μg kg^{-1}) by the Brazilian legislation in four (4.8%) of the analyzed samples. The incidence found in this study was higher than the 33, 14, 6, and 2% reported, respectively, by Lombaert et al. (2003), Kostelanská et al. (2010), Rubert et al. (2012b), Romagnoli et al. (2010).

Figure 3 displays chromatograms of cereal-based porridge samples naturally contaminated by mycotoxins.

Conclusions

An UHPLC-MS/MS method for the determination of 14 mycotoxins in cereal-based porridge destined for infant consumption was developed and validated. The sample treatment method is very useful for routine analysis, as it involves a simple simultaneous extraction/cleanup step, followed by extract concentration. The validated method was applied for the determination of target mycotoxins in 84 samples of cereal-based porridge for infant consumption. Fumonisins can be considered the main issue regarding infant exposure by mycotoxins through porridge consumption. However, aflatoxin B1, deoxynivalenol, and zearalenone were also found in concentrations exceeding the maximum permitted limit stipulated by the Brazilian legislation. The simultaneous contamination of the porridge samples by different mycotoxin classes demonstrates the importance of the analytical method developed herein.

Compliance with Ethical Standards

Conflict of Interest André Victor Sartori declares that he has no conflict of interest. Maria Heloísa Paulino de Moraes declares that she has no conflict of interest. Rosana Pereira dos Santos declares that she has no conflict of interest. Yuri Pereira Souza declares that he has no conflict of interest. Armi Wanderley da Nóbrega declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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